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Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits

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Abstract: Oxidative stress has been suggested as a mechanism underlying the costs of reproduction and life history trade-offs. Reproductive activities may lead to high production of pro-oxidants, whose activity can generate oxidative damage when not countered by adequate antioxidant defenses. Because inter-individual differences in the efficiency of the antioxidant system are influenced by an individual's diet, food availability experienced during reproduction may affect the females' antioxidant status and, in birds, their ability to transfer antioxidants into their eggs. Moreover, a female's ability to cope with oxidative stress has been suggested to influence pigment deposition in the eggshell, suggesting a possible signaling function of eggshell maculation. Here we performed a food supplementation experiment in a natural population of great tits (*Parus major*) in order to investigate how nutritional conditions experienced during the egg laying period affect the female's oxidative status and egg investment and how maternal oxidative status and egg antioxidant protection relate to eggshell pigmentation. We show that food-supplemented females had lower oxidative damage levels (ROMs) than non-food-supplemented females. Furthermore, a female's ROMs levels were negatively associated with the levels of yolk antioxidant protection in her eggs, but this negative association was only significant in non-food-supplemented females. This suggests that oxidative stress experienced during reproduction influences the allocation of antioxidants into the eggs. Moreover, we observed a positive relationship between eggshell pigment distribution and maternal and yolk antioxidant protection, suggesting that eggshell pigmentation is a cue of female (and offspring) quality.

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1 **Female oxidative status, egg antioxidant protection and eggshell**
2 **pigmentation: a supplemental feeding experiment in great tits**

3

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22 **Abstract**

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Keywords: oxidative stress, yolk antioxidants, female condition, food availability, eggshell pigmentation, costs of reproduction

Introduction

Reproduction is one of the fundamental biological processes in an individual's life, yet it is energetically and metabolically costly (Stearns 1992; Harshman and Zera 2007; Hansen et al. 2013). In oviparous species, for example, the metabolic rate of females increases by up to 27 % during egg laying (Nilsson and Råberg 2001), and both fat and protein reserves become depleted (Williams 2005). Furthermore, during incubation an up to threefold increase of female metabolic rate has been documented (Moreno and Sanz 1994; Bryant 1997; de Heij et al. 2007; Nord et al. 2010).

The metabolic acceleration occurring during reproduction can translate into higher production of reactive oxygen species (ROS) and circulating free radicals (Olsson et al. 2012). The uncontrolled activity of such molecules can cause cell damage and oxidative stress (Halliwell and Gutteridge 2007), which is defined as the imbalance between pro-oxidants and antioxidant defense in favor of the former (Sies 1991). An increase of oxidative damage and a reduction of antioxidant protection have for example been observed in response to an experimental increase of reproductive effort (Wiersma et al. 2004; Travers et al. 2010). Moreover, higher oxidative damage levels were found in breeding individuals as compared to non-breeding individuals (Costantini et al. 2014). The results of these studies suggest that oxidative stress may be a cost of reproduction (Costantini 2008; Metcalfe and Alonso-Alvarez 2010; Metcalfe and Monaghan 2013).

The antioxidant system of animals acts through different lines of defense, which involve endogenous elements (e.g. thiol groups and enzymes) and exogenous compounds acquired through the food (e.g. vitamins and carotenoids) (Catoni et al. 2008). The efficiency of the antioxidant system of an individual may thus be affected by its diet (Catoni et al. 2008; Cohen et al. 2009), in particular by the availability of proteins, which are involved in the synthesis of antioxidant compounds (Li et al. 2002; Alan and McWilliams 2013) and which can influence the activity of various antioxidant enzymes (Fechner et al. 2001). Indeed, a low-protein diet has been found to lead to increased oxidative damage and decreased antioxidant activity in both captive and wild-living animals (Feoli et al. 2006; Alan and McWilliams 2013; Giordano et al. 2015).

In birds, a female's physiological and nutritional condition can affect her ability to deposit antioxidants into the eggs (Blount et al. 2002; Saino et al. 2002; Berthouly et al. 2007; Török et al. 2007; Costantini 2010). Egg antioxidants provide protection to the developing embryo against the deleterious effects of free radicals produced during prenatal growth (Surai et al. 1996), and low levels of yolk antioxidants have been shown to impair embryo development (Wilson 1997). Furthermore, low antioxidant availability during embryonic development can negatively affect an individual's reproductive success later in life (Surai 2002), highlighting the crucial role of maternally-derived yolk antioxidants in modulating inter-individual variation in fitness.

In birds, eggshells are characterized by a variety of colour patterns created by the deposition of two main pigments: protoporphyrin, which causes red-brown eggshell colouration, and biliverdin, which causes blue-green eggshell colouration (Kennedy and Vevers 1976). Because of the antioxidant properties of biliverdin (Kaur

et al. 2003), it has been suggested that the deposition of these pigments into the eggshell may signal a female's antioxidant capacity to her mate ('sexually selected eggshell colouration hypothesis' (SSH); Moreno and Osorno 2003). Indeed, some studies found that females with a higher ability of oxidative stress resistance lay eggs with more intense blue-green coloration (Hanley et al. 2008; reviewed in Reynolds et al. 2009). Protoporphyrin, on the other hand, has pro-oxidant properties (Afonso et al. 1999), and it has been suggested that an intense red-brown shell pigmentation may be related either to higher oxidative tolerance of females capable of efficiently removing protoporphyrin via the shell, or to poor physiological condition of females experiencing high levels of circulating protoporphyrin (Moreno and Osorno 2003). However, to date no study has directly tested for a relationship between protoporphyrin-based eggshell maculation and female oxidative condition.

Here we experimentally manipulated the availability of protein-rich food shortly before and during egg laying in a natural population of great tits (*Parus major*), in order to investigate how food availability during reproduction affects female oxidative status, yolk antioxidant capacity and their relationship with eggshell pigmentation. We predicted that food-supplemented females have enhanced oxidative protection and / or reduced oxidative damage, and that females with lower oxidative stress produce eggs with a higher antioxidant content. Furthermore, based on Moreno and Osorno 2003 (see above), we predicted to find either a positive or a negative relationship between female oxidative status and eggshell maculation.

Material and Methods

Field procedures

The study was carried out between March and June 2012 in a nestbox breeding population of great tits in a forest dominantly composed of beech and spruce (Zurichbergwald, Switzerland; 47°20'08'' N, 8°30'01'' E). The nestboxes (12.5cm x 12.5cm x 26.5 cm, Type Varia; wooden nestboxes equipped with a waterproof cover) were checked every other day from March onward to monitor the progress of nest building and the beginning of egg laying. At an advanced stage of nest building, but before the first egg was laid (mean \pm 1 standard deviation (SD) = 7.1 \pm 4.7 days before egg laying), we randomly assigned nestboxes to the food supplementation (F) or to the control group (NF). F-nests were supplemented with 15g of diptera larvae (*Sarcophaga* spp), which represent ca. 80% of the daily energy need of adult great tits (Gibb 1957), every other day until the clutch was completed. The NF nests were visited and treated as the F nests, but they did not receive extra food. All experimental nests were first breeding events.

In great tits the time from the beginning of ovum development to its deposition is approximately 4 days (Perrins 1996). Consequently, food-supplemented females experienced increased food availability before the formation of the first egg and during the whole egg laying period. In order to ensure that only the breeding pair had access to the supplemented food, we placed the larvae in a plastic cup, which was attached to the inside wall of the nestbox, and not visible from the outside. All larvae were eaten within two days (MG pers. obs.). We are aware of the possibility that males may have eaten part of the supplementary food. However, the significant effect of the food treatment on female oxidative condition (see Results) and egg traits (Giordano et al. 2015) demonstrates that females ate a substantial part of the supplemental food and that this influenced their egg provisioning.

We marked newly laid eggs with a non-toxic marker, and collected the fourth

egg of each clutch on the day it was laid. On the same day, the egg was weighed, and the yolk was separated from the albumen, weighed, and frozen at -80°C until analysis. Only a subsample of the collected eggs could be used for antioxidant analyses (see below) because some eggs were damaged during transport..

The mean clutch size (\pm SD) in our study population was 8.2 (\pm 1.3) eggs. The fourth egg is therefore one of the middle eggs of the laying sequence. In great tits, variation in the levels of yolk antioxidants is low within clutches, and yolk antioxidant concentrations do not vary with laying sequence (Isaksson et al. 2008; Remeš et al. 2011; see also Tschirren et al. 2004; Postma et al 2014 for other egg components). Therefore the antioxidant capacity of the fourth egg can be considered representative for the antioxidant capacity of the clutch.

At clutch completion, a photo of all eggs was taken. Thereto, the eggs were removed from the nest, placed on a plate, and photographed in a standardized way using a Canon EOS 1000D digital camera (Canon Inc., Tokyo, Japan) from a distance of 10 cm. Millimeter paper was photographed together with the eggs to allow for calibration.

Breeding females (F: $N = 31$; NF: $N = 39$) were caught when their nestlings were 10 days old. Females are prone to desert their nest when disturbed during the egg laying or incubation period. For this reason, we caught females only during the nestling stage. Females were weighed with a Pesola spring balance (accuracy of 0.25 g) and a small blood sample (approximately 100 μ l) was taken from their brachial vein. The blood was kept cool until centrifugation (10,621x g for 10 minutes), which occurred on the same day. After centrifugation, we separated plasma and red blood cells and stored both at -80° C until oxidative stress analyses (see below), which were performed within 4 months after blood sampling.

Female non-enzymatic plasmatic antioxidant capacity

We measured the females' non-enzymatic plasmatic antioxidant capacity (OXY) (F: $N = 31$; NF: $N = 39$) using the OXY- Adsorbent test (Diacron International, Grosseto, Italy) following Costantini et al. (2011). This assay quantifies the ability of a biological matrix (plasma or yolk) to oppose the oxidizing action of hypochlorous acid, one of the strongest oxidant agents in biological systems (Halliwell and Gutteridge 2007). In short, plasma (2 μ l) was diluted 1 : 100 (vol : vol) with distilled water. Then 2 μ l of the diluted plasma was added to 200 μ l of a titred HOCl solution and incubated for 10 min at 37 °C. At the end of incubation, 2 μ l of chromogen were added and the absorbance was read at a wavelength of 490 nm (SpectraMax 340PC³⁸⁴ Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Measurements are expressed as mM of HOCl neutralized. Samples were run in duplicate. Repeatability of OXY measures was high ($r = 0.93$, $P < 0.001$, $N = 70$). The interassay coefficient of variation was 5.2% and the intrassay coefficient of variation was 3.7%.

Female glutathione peroxidase activity

Glutathione peroxidase activity (GPX) in the females' red blood cells (F: $N = 31$; NF: $N = 39$) was measured with the Ransel assay (Randox Laboratories, Crumlin, UK) (Paglia and Valentine 1967) according to the manufacturer's instructions. GPX is an antioxidant enzyme which, using thiols as cofactors, catalyzes the reduction of harmful oxidant agents (Arthur 2000). The Ransel assay assesses the ability of GPX to catalyze the neutralization of cumene hydroperoxide via oxidation of glutathione. Briefly, red blood cell samples were diluted 1 : 40 (vol : vol) with the dilution agent provided with the kit. 200 μ l of reagent and 8 μ l of cumene hydroperoxide were added

to 4 µl of the diluted sample, and, after one and three minutes, absorbance was read at a wavelength of 340 nm (ThermoFisher, Vantaa, Finland). GPX activity was calculated following the manufacturer's instructions: $[(\text{Abs } 1\text{min} - \text{Abs } 3\text{min}) / 2] * 15'873$. To control for differences in red blood cell concentrations among samples, we measured the protein content using the Bio-Rad Bradford Protein assay kit (Bio-Rad Laboratories, Inc., CA, USA) according to the manufacturer's instructions. The results are expressed as units of GPX per mg proteins. Samples were run in duplicate. Repeatability of GPX activity measures was high ($r = 0.97$, $P < 0.001$, $N = 70$). The interassay coefficient of variation was 9.7% and the intraassay coefficient of variation was 5.3%.

Female red blood cell thiols

We measured thiol-containing compounds in the females' red blood cells (F: $N = 31$; NF: $N = 39$) using the -SHp test (Diacron International, Grosseto, Italy) according to previously published protocols (Costantini et al. 2011). Thiols are antioxidant compounds characterized by a sulfhydryl group (-SH), which reacts with, and scavenges, free radicals. Glutathione is one of the major thiols occurring in animal cells (Bindoli et al. 2008). For the assay, red blood cells samples were diluted 1 : 200 (vol : vol) with distilled water. 12.5 µl of diluted red blood cells were added to a sulfate buffer (pH 7.6) and let incubate for 3 min at room temperature. After incubation, absorbance was read at 405 nm (ThermoFisher, Vantaa, Finland). 5 µl of chromogen (purchased with the kit) was added and samples were incubated for 5 min at room temperature. At the end of incubation, the absorbance was read again at 405 nm (ThermoFisher, Vantaa, Finland). Results were expressed as $\mu\text{mol l}^{-1}$ of -SH groups per mg proteins. Samples were run in duplicate. Red blood thiols measures

were highly repeatable ($r = 0.97$, $P < 0.001$, $N = 70$). The interassay coefficient of variation was 6.7% and the intraassay coefficient of variation was 6.4%.

Because several antioxidant capacity markers were significantly correlated (Pearson product-moment correlation: GPX / Thiols: $r = 0.167$, $P = 0.041$, $N = 70$; GPX / OXY: $r = 0.185$, $P = 0.023$, $N = 70$; Thiols / OXY: $r = -0.084$, $P = 0.303$, $N = 70$), we performed a Principal Component Analysis and used the first two principle components (antioxidant capacity biomarkers PC1 and PC2) in the statistical analyses. The first principal component (antioxidant capacity PC1) explained 41.8 % of the variation in female antioxidant status. Antioxidant capacity PC1 describes the component of the female's antioxidant system involving thiols and GPX activity (enzymatic-thiolic component) (Loadings: GPX: 0.793, Thiols: 0.792, OXY: 0.305). The second principal component (antioxidant capacity PC2) explained further 34.4 % of the variation in female antioxidant status. Antioxidant capacity PC2 describes the non-enzymatic plasmatic antioxidant capacity (Loadings: GPX: 0.222, Thiols: -0.234, OXY: 0.963). Antioxidant capacity biomarkers were not normally distributed, thus they were log transformed before performing the Principal Component Analysis.

Female oxidative damage

We estimated the females' plasma concentration of oxidative damage (F: $N = 31$; NF: $N = 39$) using the colorimetric dROMs test (Diacron International, Grosseto, Italy). This assay measures intermediate oxidative damage molecules (mostly hydroperoxides; Alberti et al. 2000). The assay was performed following previously published protocols (Costantini et al. 2011). In short, plasma (8 μ l) was diluted with 200 μ l of an acetate buffer (pH 4.8) and an aromatic alkyl-amine (chromogen). The samples were incubated for 75 min at 37 °C. At the end of the incubation, the samples

were centrifuged (17,950 x g for 2 min). The supernatants (190 µl) were pipetted in a microplate and the absorbance was read at a wavelength of 505 nm (ThermoFisher, Vantaa, Finland). Results are expressed as mM of H₂O₂ equivalents. Samples were run in duplicate. The repeatability of ROMs measures was high ($r = 0.98$, $P < 0.001$, $N = 70$). The interassay coefficient of variation was 10.4%, and the intraassay coefficient of variation was 5.4%. Oxidative damage measures (ROMs) were not normally distributed, thus they were log transformed prior to statistical analyses.

Yolk antioxidant capacity

We measured the yolk antioxidant capacity (F: $N = 14$, NF: $N = 11$) using the OXY-Adsorbent test (Diacron International, Grosseto, Italy) following previously published protocols (Costantini 2010). In short, we extracted the antioxidants in the yolk by diluting an aliquot of yolk (60 mg) with 5 ml of distilled water, and vortexing and sonicating this solution for 10 min. The dilution of yolk with water produces an emulsion in which all yolk antioxidants (e.g. carotenoids, vitamin A, vitamin D, vitamin E) are embedded. We then performed the same procedure for a second aliquot of yolk, to which we added 15 µl of a solution of known concentration of OXY standard. The difference in concentration between the second yolk aliquot and the OXY standard was divided by the concentration of the first yolk aliquot to determine extraction efficiency, which was (mean \pm SD) $95 \pm 1.9\%$. The yolk solution was centrifuged (10,621x g for 2 minutes) and the supernatant was diluted 1 : 4 (vol : vol) with distilled water. 2 µl of the diluted yolk solution was then incubated with 200 µl of titred HOCl for 10 mins at 37°C. At the end of the incubation, 2 µl of chromogen (purchased with the kit) was added, and the absorbance was spectrophotometrically read at a wavelength of 490 nm (SpectraMax 340PC³⁸⁴ Microplate Reader, Molecular

Devices, Sunnyvale, CA, USA). Measurements are expressed as μM of HOCl neutralized per mg yolk. Samples were run in duplicate. Yolk OXY measures were highly repeatable ($r = 0.98$, $P < 0.001$, $N = 25$). The interassay coefficient of variation was 7.4%, and the intraassay coefficient of variation was 5.5%. Yolk OXY values were not normally distributed, thus they were log transformed before performing statistical analyses. Since it was not possible to perform the OXY assay on some eggs, the yolk OXY sample size is lower than total number of eggs collected.

Eggshell pigmentation

Great tits lay white eggs maculated with protoporphyrin-based red-brown spots. Eggshell maculation was scored on the photographed side of the egg using the criteria described in Gosler (2000) (F: $N = 209$ eggs of 31 clutches, NF: $N = 271$ eggs of 39 clutches). Pigment intensity (I) was scored from 1 (pale pigments) to 5 (dark pigments). Pigment distribution (D) was scored from 1 (pigments concentrated on one part of the egg) to 5 (pigments evenly distributed). Spot size (S) was scored from 1 (small spots) to 3 (big spots). Zero scores were not assigned. All eggs were scored by the same person (MG). The fourth egg, collected to measure yolk antioxidant capacity, was not scored. In order to calculate repeatability of eggshell pigmentation scores, a subset of eggs was scored twice. Repeatability was I: $r = 0.67$, $P < 0.001$, $N = 130$; D: $r = 0.72$, $P < 0.001$, $N = 130$; S: $r = 0.51$, $N = 130$. The mean I, D, S per clutch was calculated and used for subsequent analyses (within-clutch repeatability: I: $r = 0.87$, $P < 0.001$, $N = 480$; D: $r = 0.79$, $P < 0.001$, $N = 480$; S: $r = 0.51$, $P < 0.001$, $N = 480$).

Statistical analyses

Firstly, we used general linear models to test for effects of the food treatment on female antioxidant capacity PC1 and PC2, female oxidative damage (ROMs), female body mass, egg mass, yolk mass, yolk antioxidant capacity, eggshell pigmentation (intensity, distribution and spot size) and clutch size.

Secondly, we fitted a general linear model to assess whether food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, and their two-way interactions affected yolk antioxidant capacity.

Finally, we used general linear models to test for the effects of the food treatment, female antioxidant capacity PC1 and PC2, female oxidative damage, yolk antioxidant capacity, and their two-way interactions on eggshell pigmentation (intensity, distribution and spot size).

Final models were obtained with a stepwise backward procedure. Non-significant interactions and factors ($P > 0.05$) were removed from the models, starting with the least significant interaction term. Residuals from all the models were checked for homoscedasticity and normality. No variance inflation factors (VIF) equal to or higher than 2 were identified (Marques de Sá 2007). Analysis were performed in JMP 10 (SAS Institute 1989-2007).

Results

Effect of food treatment on female oxidative status

Food-supplemented females had significantly lower levels of oxidative damage than non-supplemented females (ROMs: $F_{1,68} = 5.976$, $P = 0.017$; Fig.1). No effect of the food supplementation on the females' enzymatic-thiolic antioxidant capacity ($F_{1,68} = 1.079$, $P = 0.303$) or non-enzymatic antioxidant capacity ($F_{1,68} = 1.934$, $P = 0.169$)

was observed. No significant effect of the food supplementation on female body mass was found ($F_{1,68} = 1.562$, $P = 0.216$).

Effect of food treatment on yolk antioxidant status and reproductive investment

The food supplementation did not significantly affect the antioxidant capacity of the eggs ($F_{1,23} = 0.298$, $P = 0.591$), egg mass ($F_{1,63} = 0.114$, $P = 0.726$), yolk mass ($F_{1,63} = 0.421$, $P = 0.519$), or clutch size ($F_{1,68} = 2.533$, $P = 0.116$). Furthermore, there was no treatment effect on eggshell pigmentation (intensity: $F_{1,68} = 0.464$, $P = 0.498$; spot size: $F_{1,68} = 2.304$, $P = 0.134$; distribution ; $F_{1,68} = 0.488$, $P = 0.491$).

Relationship between female oxidative status and yolk antioxidant status

The females' levels of oxidative damage (ROMs) were negatively related to the antioxidant capacity of their eggs ($F_{1,19} = 11.207$, $P = 0.003$). Moreover, there was a significant interaction effect between the food treatment and the females' levels of oxidative damage on yolk antioxidant capacity ($F_{1,19} = 11.203$, $P = 0.003$; Fig. 2). The decrease of the egg antioxidant capacity with increasing ROMs levels of the mother was pronounced in non-supplemented ($b = -0.165$; $F_{1,9} = 21.437$, $P = 0.012$), but weak and non-significant in supplemented females ($b = -0.018$; $F_{1,12} = 0.096$, $P = 0.762$). No relationship between egg antioxidant capacity and female enzymatic-thiolic antioxidant capacity ($F_{1,19} = 0.034$, $P = 0.855$) or non-enzymatic antioxidant capacity ($F_{1,19} = 1.801$, $P = 0.195$) was observed.

Relationships among eggshell pigmentation, female oxidative status and yolk antioxidant status

Eggshell pigment distribution was significantly positively related to female enzymatic-thiolic antioxidant capacity ($b = 0.505$; $F_{1,22} = 8.495$, $P = 0.008$; Fig. 3a). Females with a higher blood antioxidant capacity laid eggs with a more homogenous spot distribution. Similarly, pigment distribution was significantly related to the antioxidant capacity of the yolk ($b = 0.391$; $F_{1,22} = 5.09$, $P = 0.034$; Fig. 3b). Eggs with a higher yolk antioxidant capacity had a more homogenous shell spot distribution. No relationships between eggshell pigment distribution and female non-enzymatic antioxidant capacity ($F_{1,21} = 1.555$, $P = 0.226$) or female oxidative damage ($F_{1,20} = 0.192$, $P = 0.666$) were observed.

No relationships between pigmentation intensity and female antioxidant capacity (enzymatic-thiolic antioxidant capacity: $F_{1,21} = 1.350$, $P = 0.258$; non-enzymatic antioxidant capacity: $F_{1,19} = 0.001$, $P = 0.977$), female oxidative damage ($F_{1,22} = 1.359$, $P = 0.256$), or yolk antioxidant capacity ($F_{1,23} = 1.222$, $P = 0.281$) were observed.

Similarly, no relationships between spot size and female antioxidant capacity (enzymatic-thiolic antioxidant capacity: $F_{1,66} = 0.924$, $P = 0.339$; non-enzymatic antioxidant capacity: $F_{1,64} = 0.052$, $P = 0.821$), female oxidative damage ($F_{1,65} = 0.121$, $P = 0.729$), or yolk antioxidant capacity ($F_{1,19} = 0.034$, $P = 0.855$) were detected. None of the interactions between food treatment and female or egg traits ($P > 0.091$ in all cases) significantly affected eggshell pigmentation (D, I, S) and they were therefore not retained in the final models.

Discussion

Here we experimentally tested in a wild bird population how nutritional conditions experienced during the egg laying period affect female oxidative status and the allocation of antioxidants into the eggs. Moreover, we tested the hypothesis that protoporphyrin-based eggshell pigmentation relates to a female's oxidative condition and her antioxidant investment into the eggs.

The level of oxidative damage (ROMs) in the females' circulation during the nestling rearing period was strongly influenced by the food treatment. Females that received extra food during the egg laying period had less oxidative damage than non-supplemented females.

Whereas circulating levels of oxidative damage were reduced when females received extra food, we observed no difference in antioxidant protection between treatment groups. One explanation for these findings is that the food supplementation led to a reduction in the production of oxidizing agents. Given that the extra food was provided within the nest, supplemented females may, for example, have reduced their flying and food searching time. Indeed, physical activity can cause an imbalance in the levels of fatty acids, with an increase of unsaturated fatty acids, molecules susceptible to oxidative damage (Porter et al. 1995; Nikolaidis and Mougios 2004). Furthermore, the contraction of skeletal muscles occurring during flying can result in an accelerated production of oxidizing agents, such as superoxide (Powers and Jackson 2008).

An alternative explanation is that the food supplementation might not have reduced the production of oxidizing agents, but it may have boosted an (unmeasured) component of the antioxidant machinery, as for example uric acid (Alan and McWilliams 2013) or catalase (Rindler et al. 2013). In fact, protein levels in the diet have been shown to affect a variety of antioxidant pathways (e.g. enzymatic activity

(Fechner et al. 2001), production of uric acid (Alan and McWilliams 2013) and synthesis of thiol-containing compounds (Li et al. 2002)). Moreover, we cannot exclude the possibility that the food supplementation induced short-lasting beneficial effects on the measured antioxidant components, not persisting for long enough to be detected. Finally, the food supplementation might have affected the birds' cell membrane composition, making them less susceptible to oxidative damage (Hulbert et al. 2007).

Although the mechanism behind the lower levels of oxidative damage observed in supplemented females remains currently unknown, our data clearly show that benefits of having extra food available during egg laying can carry over and can still be detected during the nestling rearing phase. These results imply that an individual's oxidative balance is sensitive to relatively short-term changes in territory quality, meaning that an increase in breeding territory quality can translate in a reduction in the oxidative cost of reproduction (van de Crommenacker et al. 2010).

Previous studies on captive and wild birds have linked an increased reproductive effort with a reduced ability to neutralize free radicals. Captive zebra finches (*Taeniopygia guttata*) females, for example, showed a reduced red blood cell antioxidant capacity and lower antioxidant enzymes activity when rearing enlarged broods (Alonso-Alvarez et al. 2004; Wiersma et al. 2004). Similarly, great tits rearing an experimentally enlarged brood had a lowered resistance to oxidative stress (Christe et al. 2012). We found that food-supplemented and non-supplemented females had a similar level of reproductive investment (in terms of egg mass, yolk mass, yolk antioxidant capacity and clutch size; see also Giordano et al. 2014), but supplemented females experienced significantly lower levels of oxidative stress. This suggests that the supplemented food allowed females to counter the oxidative costs of reproduction,

and that extra nutrients derived from the supplementation were used for self-maintenance, rather than for increasing reproductive investment.

Whereas the food supplementation did not directly influence yolk antioxidant deposition, we found that it influenced the relationship between a female's levels of oxidative damage and the yolk antioxidant capacity of her eggs. Females with higher plasma oxidative damage levels deposited lower amounts of antioxidants in their eggs, suggesting that oxidative stress may constrain the female's reproductive investment. This negative association between female oxidative damage and egg antioxidant investment was evident only in non-supplemented females, whereas supplemented females were able to sustain a high deposition of yolk antioxidants, regardless of their circulating levels of oxidative damage. Being able to sustain high levels of antioxidant deposition may be advantageous because low levels of yolk antioxidants can impair embryo development (Wilson 1997), and a reduced yolk antioxidant deposition may negatively affect the nestlings' survival prospects (McGraw et al. 2005; Marri and Richner 2014).

It has been proposed that females may signal their oxidative condition to the male by modulating the deposition of eggshell pigments, thereby eliciting a higher paternal effort (Moreno and Osorno 2003). Most studies that tested this hypothesis focused on biliverdin-based eggshell colours (e.g. Morales et al. 2008), whereas the signaling function of protoporphyrin-based eggshell pigmentation is less well understood.

Support for a potential signaling function of protoporphyrin-based maculation comes from a recent study in great tits, which showed that the intraclutch increase in pigmentation darkness was positively related to female quality (i.e. body size and presence of red cells lysis) (De Coster et al. 2013). In contrast with this result,

Martínez de la Puente et al. (2007) showed a negative relationship between eggshell spottiness and female condition (i.e. immunoglobulin levels) in blue tits (*Cyanistes caeruleus*), a species closely related to great tits. However, these studies did not test for a relationship between eggshell pigmentation and female oxidative condition.

In our study no relationship between pigmentation intensity or spot size and female oxidative status was detected. But interestingly, we found a significant relationship between the distribution of protoporphyrin-based pigments on the eggshell and both yolk and female antioxidant capacity. Eggs with high yolk antioxidant capacity and eggs produced by females with high enzymatic-thiolic antioxidant capacity had more homogenously distributed spots. To the best of our knowledge, this is the first observation of a relationship between female oxidative status and eggshell pigmentation in a species with protoporphyrin-based eggshell coloration.

In blue tits, another species with protoporphyrin-colored eggs, males were found to adjust their parental effort according to eggshell pigment distribution (Sanz and García-Navas 2009), with an increase of the daily paternal provisioning rate for nestlings hatched from eggs with more evenly distributed shell spots. It suggests that males are able to discriminate eggshell patterns, also in cavities and nestboxes, and that eggshell pigment distribution influences paternal investment.

However, besides a potential signaling function there are alternative explanations for the relationship between eggshell pigment distribution and female and egg oxidative status observed in our study. For example, eggshell pigmentation can influence eggshell strength (Gosler et al. 2005), and females in good physiological condition might simply be able to produce more robust eggs. Although the possible physiological mechanisms and costs behind this process are not known,

our results suggest that females with high antioxidant capacity may be able to deploying protoporphyrin more widely through the shell, with possible benefits for shell stability. In order to disentangle the function behind eggshell maculation, future work should simultaneously and experimentally test the alternative hypotheses (i.e. signaling vs. structural function).

In conclusion our results show that (1) food availability experienced during the laying period can influence the oxidative cost of reproduction in great tit females and, that (2) the female's oxidative status and her nutritional condition influence the deposition of antioxidants into the eggs in an interactive way. Moreover, we found that (3) eggshell spot distribution relates to female and yolk antioxidant status, thereby supporting the hypothesis that eggshell maculation is a cue of female quality.

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Ethical standards

All procedures were conducted under license from the Veterinary Office of the Canton of Zurich, Switzerland (195/2010) and the Federal Office for the Environment, Switzerland (324).

494 **Conflicts of interest**

495 The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Effect of the food treatment on plasma oxidative damage levels (ROMs; mM H₂O₂ equivalents) in food supplemented (filled circle; *N* = 31) and non-supplemented (open circle; *N* = 39) female great tits. Means \pm 1 SE are shown

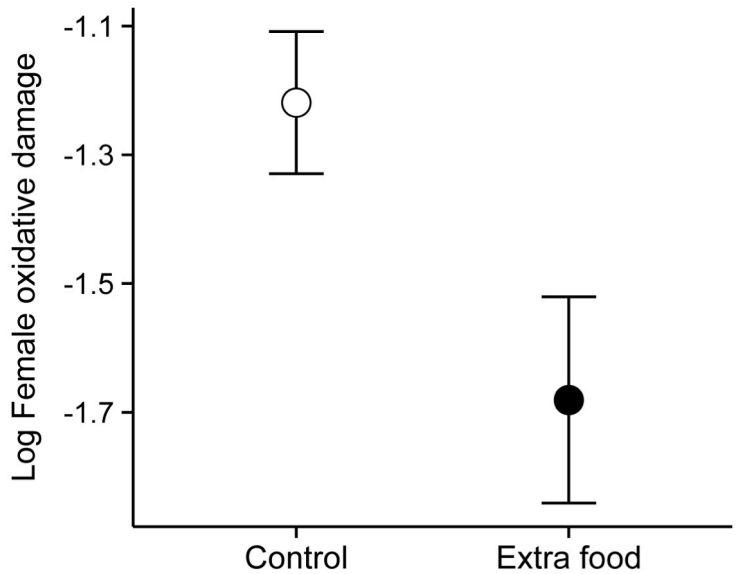
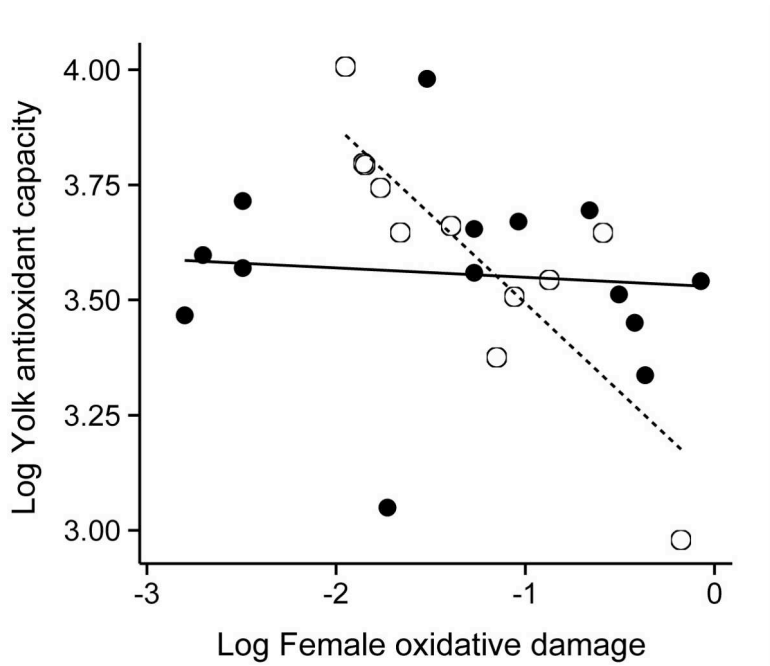


Fig. 2 Relationship between female plasma oxidative damage (ROMs; mM H₂O₂ equivalents) and yolk antioxidant capacity (yolk OXY; μ M HOCl neutralized mg⁻¹ yolk) in food supplemented (filled circle, solid line; *N* = 14) and non-supplemented (open circle, dotted line; *N* = 11) females



697 **Fig. 3** Relationships between eggshell pigmentation distribution and (a) female
 698 enzymatic-thiolic antioxidant capacity (PC1) and (b) yolk antioxidant capacity (OXY;
 699 $\mu\text{M HOCl}$ neutralized mg^{-1} yolk)

